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Sulfatase activity assay using an activity-based probe by generation of *N*-methyl isoindole under reducing conditions

Hey Young Yoon, Jong-In Hong*

Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-747, Republic of Korea

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ABSTRACT

Sulfatases catalyze the hydrolysis of sulfate esters that are present in a range of biomolecules. This is an important step in several biological processes such as cellular degradation, hormone regulation, and cell signaling. We have developed a new activity-based sulfatase probe (probe 1) that generates a fluorescent *N*-methylisoindole upon hydrolysis by sulfatase. Because of the autoxidation of *N*-methylisoindole, the sulfatase activity was also tested under reducing conditions, containing either glutathione (GSH) or tris(2-carboxyethyl)phosphine (TCEP), exhibiting little change in kinetic parameters compared to non-reducing conditions. Probe **1** displayed reasonable kinetic parameters under both non-reducing and reducing conditions, among which the use of Tris buffer and Tris buffer containing GSH appeared to be appropriate conditions for inhibitor screening. Probe **1** showed stronger intensity upon treatment with sulfatase under neutral conditions than under acidic conditions, but it still has limitations in the selectivity for a specific sulfatase. Nevertheless, the fluorescent signal generated as a result of the release of *N*-methylisoindole after treatment of probe **1** with sulfatase provides a new assay for measuring sulfatase activity that could be adapted for high throughput screening.

Introduction

In many biological systems, the sulfation state of biomolecules is important in determining their function. Sulfation state is regulated by sulfatases (EC 3.1.5.6) which catalyze the cleavage of sulfate esters present in biomolecules such as carbohydrates, steroids, and proteins [1–3]. Human sulfatases are broadly classified based on their subcellular location. The three classes are, lysosomal sulfatases, non-lysosomal sulfatases, and extracellular sulfatases. Sulfatases in each subcellular location play important roles in many biological processes including cellular degradation, hormone regulation, and cell signaling. In breast carcinoma cells, for example, it has been reported that the mRNA level is increased but the activity of steroid sulfatases is decreased [4,5]. In order to deepen our understanding of sulfatase biology, the development of probes capable of measuring sulfatase activity is required. Optical probes have proven to be powerful tools for measuring sulfatase activity and these assays have contributed to the diagnosis and treatment of sulfatase-related diseases.

In general, sulfatase activity assays have been performed using *p*-nitrophenyl sulfate (*p*-NPS) or 4-methylumbelliferyl sulfate (4-MUS). However, these sulfatase substrates have slow reaction rates and lack sensitivity [6–8]. Several other activity-based sulfatase probes have been reported. These are comprised of luminophores and a sulfate group, and they exhibit an optical change after cleavage of the sulfate ester by sulfatases [9–13]. These probes have better kinetics and increased sensitivity compared to *p*-NPS and 4-MUS [9–14], and they were able to discriminate mycobacterium strains through individual sulfatase activity patterns [13].

Here, we introduce a new activity-based probe for measuring sulfatase activity assay using the formation of fluorescent *N*-methylisoindole [15]. Based on the ability of sulfatase to cleave sulfate groups, we designed a pro-fluorescent probe (probe **1**) which consists of a phenyl sulfate moiety as a substrate and 2-formyl benzylcarbamate moiety as a signaling unit. When the sulfate group of probe **1** is cleaved by sulfatase, self-immolation and intramolecular cyclization occur, resulting in the generation of a fluorescent *N*-methylisoindole (Scheme 1). Isoindole derivatives has been reported to be unstable and decompose easily, resulting in reduced fluorescence intensity [16–21]. To avoid this reduction in fluorescence intensity, we also examined sulfate ester hydrolysis under reducing conditions, namely in the presence of glutathione

* Corresponding author.
E-mail address: jihong@snu.ac.kr (J.-I. Hong).

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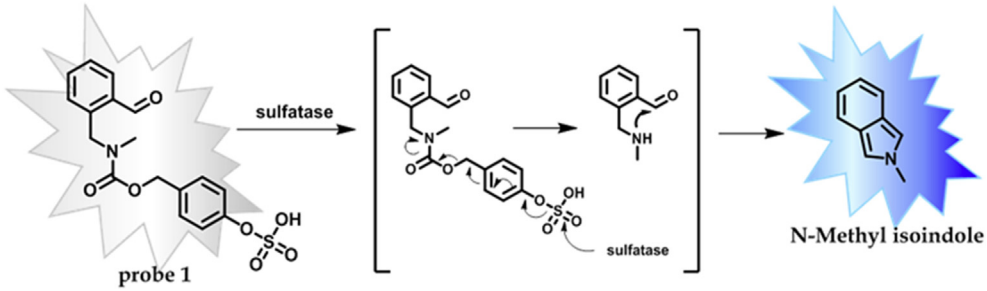

Scheme 1. Probe **1** for sulfatase activity assay.

(GSH) or tris(2-carboxyethyl)phosphine (TCEP).

Materials and methods

Synthesis of probe 1

*Synthesis of neopentyl(4-((((4-nitrophenoxy)carbonyl)oxy)methyl) phenyl) sulfate (**8**)*

Compound **7** was prepared according to a published procedure [22] and its synthesis is described in more detail in the Data in Brief [23]. To a solution of 4-nitrophenyl chloroformate (640 mg, 1.1 equiv.) in anhydrous THF was added pyridine (257 μL, 1 equiv.) at 0 °C. After stirring for 20 min, a solution of compound **7** (970 mg, 3.54 mmol) in anhydrous THF was added dropwise over a 10-min period, and the mixture was allowed to warm up to room temperature [24]. Stirring was continued at room temperature for 16 h and then the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate and washed with saturated aqueous NH_4Cl solution several times and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (chloroform:acetone = 100:1) to yield compound **8** (784 mg, 1.78 mmol, 50% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.03 (9H, s), 4.13 (2H, s), 5.32 (2H, s), 7.38 (2H, d, J = 8.5 Hz), 7.41 (2H, d, J = 9.0 Hz), 7.52 (2H, d, J = 8.6 Hz), 8.30 (2H, d, J = 8.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.86, 31.90, 69.75, 83.69, 115.331, 121.43, 121.81, 125.28, 125.46, 130.30, 133.64, 145.39, 150.48, 155.45; HRMS (FAB): m/z calcd. for [$C_{19}H_{21}NO_9S + Na^+$] 462.0835, found 462.0838.

*Synthesis of methyl 2-(dimethoxymethyl)benzoate (**3**)*

To a solution of methyl 2-formylbenzoate (**2**) (1.62 g, 9.87 mmol) in methanol (20 mL) at 0°Cunder N_2 was added 1 M $TiCl_4$ (1 mL, 0.1 equiv.) solution in CH_2Cl_2 in a single step. After stirring for 30 min, the resulting mixture was treated with triethylamine (3 mL, 10 equiv.), and the mixture was stirred for an additional 3 h at room temperature. After removal of all volatile compounds under reduced pressure, the residue was dissolved in ethyl acetate and washed with brine 3 times, dried over Na_2SO_4 and concentrated. The crude residue was purified by silica gel column chromatography (hexane:ethyl acetate = 5:1) to yield compound **3** (1.8 g, 8.56 mmol, 87% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.39 (6H, s), 3.93 (3H, s), 6.08 (1H, m), 7.40 (1H, t, J = 7.5 Hz), 7.53 (1H, t, J = 7.6 Hz), 7.76 (1H, d, J = 7.7 Hz), 7.82 (1H, d, J = 7.6 Hz).

*Synthesis of 2-(dimethoxymethyl)benzaldehyde (**4**)*

To a suspension of lithium aluminum hydride (250 mg, 2 equiv.) in anhydrous THF compound **3** (900 mg, 4.3 mmol) dissolved in anhydrous THF was slowly added at 0 °C under N_2. The reaction mixture was stirred at room temperature for 5 h, and was then cooled to 0 °C and quenched with a 1 M aqueous NaOH solution. The mixture was dried over Na_2SO_4 and filtered through a Celite pad. The filtrate was concentrated *in vacuo*. The resulting product was used for the next synthetic step without further purification.

To a solution of the reduction product (653 mg, 3.59 mmol) dissolved in CH_2Cl_2 was added MnO_2 (3.7 g, 10 equiv.). After being stirred at room temperature overnight, the mixture was filtered with Celite and a silica pad. The filtrate was concentrated *in vacuo* and the residue was purified by silica column chromatography (hexane:ethyl acetate = 3:1) to yield compound **4** (540 mg, 2.98 mmol, 69% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.42 (6H, s), 5.90 (1H, s), 7.52 (1H, t, J = 7.3 Hz), 7.62 (1H, t, J = 7.4 Hz), 7.70 (1H, d, J = 7.5 Hz), 7.95 (1H, d, J = 7.5 Hz), 10.46 (1H, s).

*Synthesis of 4-((((2-(dimethoxymethyl)benzyl)(methyl)carbamoyl) oxy)methyl)phenyl neopentyl sulfate (**6**)*

To a solution of compound **4** (540 mg, 2.98 mmol) in methanol was added a 2 M solution of methylamine in THF (4 mL, 2 equiv.). The resulting mixture was stirred at room temperature overnight, cooled to 0 °C and treated with sodium borohydride (570 mg, 5 equiv). The mixture was stirred for 1 h and then quenched with water. After removal of all volatile compounds under reduced pressure, the residue was dissolved in ethyl acetate and washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated *in vacuo*. Product **5** was used for the next synthetic step without further purification (219 mg).

To a solution of compound **5** (89 mg, 0.46 mmol) in THF were added triethylamine (192 μL, 3 equiv.) and compound **8** (200 mg, 1 equiv.). The mixture was stirred at room temperature overnight and concentrated *in vacuo*, and the residue was dissolved in ethyl acetate and washed with aqueous $NaHCO_3$ solution and saturated aqueous NH_4Cl solution, dried over Na_2SO_4 and concentrated. The residue was purified by silica column chromatography (chloroform:acetone = 50:1) to yield compound **6** (107 mg, 0.22 mmol, 47% yield). ^{1}H NMR (300 MHz, CDCl3) δ 1.02 (9H, s), 2.92 (3H, d, J = 24 Hz), 3.31(6H, d, J = 10 Hz), 4.10 (2H, s), 4.69 (2H, s), 5.19 (2H, d, J = 15.6 Hz) 5.41 (1H, d, J = 23 Hz), 7.16–7.23 (2H, br), 7.31 (3H, m), 7.46 (1H, m), 7.55 (1H, s).

*Synthesis of 4-((((2-formylbenzyl)(methyl)carbamoyl)oxy)methyl) phenyl hydrogen sulfate (probe **1**)*

To a solution of compound **6** (57 mg, 0.12 mmol) in acetone was added *p*-toluenesulfonic acid monohydrate (6.5 mg, 0.3 equiv.) and the resulting solution was stirred at room temperature for 2 h. Acetone was removed *in vacuo* and the residue was purified by silica column chromatography (chloroform:acetone = 30:1). The resulting product (50 mg, 0.11 mmol) and sodium azide (10 mg, 1.2 equiv.) were then dissolved in DMF. The resulting solution was heated with stirring at 70 °C overnight and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (CH_2Cl_2:methanol = 10:1) and yielded probe **1** as the sodium salt (37 mg, 0.098 mmol, 88% yield). ^{1}H NMR (300 MHz, CD_3OD) δ 2.95 (3H, s), 4.96 (2H, s), 5.12 (2H, d, J = 21 Hz) 7.21–7.39 (5H, m), 7.52 (1H, t, J = 7.3 Hz), 7.60 (1H, s), 7.92 (1H, d, J = 7.1 Hz), 10.15 (1H,